#28
5-30-03

Practitioner's Docket No. 700157-046793 *PATENT*

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Fine et al.

Application No.: 09/269,321 Group No.: 1636

Filed: September 13, 1999 Examiner: W. Sandals

For: METHOD OF TARGETING MALIGNANT CELLS USING AN E2F RESPONSE PROMOTER

DECLARATION OF DR. WILLIAM KAELIN

I William Kaelin hereby declare as follows:

1. I am a co-inventor of the above-described application.

2. I am a Professor of Medicine at Harvard Medical School, a physician at the Dana-Farber Cancer Institute, Inc. and a member of the Howard Hughes Medical Institute. A copy of my CV is attached hereto.

3. I am aware of the Office Action issued on November 19, 2002 in the above-described matter and am familiar with the references discussed in that Office Action.

4. The Examiner addressed Raj, G.V., et al, Oncogene 12:1279-1288 (1996) and a PCT published application WO94/18992.

5. I do not believe that these references in any way teach or suggest our method of selectively expressing a gene in a malignant cell, by determining whether the malignant cell expresses sufficient E2F to cause expression of a gene operably linked to an E2F responsive promoter. Rather, these references consider the role of E2F as a simply off/on trigger for gene expression. It was known that $_{p}$RB negatively regulates E2F and that this inhibition is relieved (under physiological conditions) by phosphorylation of $_{p}$rB which occurs at or near the time cells

enter S-phase (See Figure 1). For example, this is shown in Raj at page 279, second column, where it states:

> The activity of E2F1 appears to be regulated by its interaction with various cellular proteins, including pRb. pRb binds directly to E2F1 and inhibits its transactivation (Chellapan et al, 1991; Helin et al., 1993; Hiebert et al., 1992; Weintraub et al., 1992). In addition to pRb, E2F1 also forms complexes with p107, cyclins A and E, and cyclin dependent kinases (cdk). The **association of E2F1** with these various cellular proteins **appears to be cell-cycle regulated**, with the appearance of distinct complexes at precise states of the cell cycle (Nevins, 1994). [emphasis added]

6. Thus, Raj is talking about a binary system, with E2F either "on" (when not bound to $_{p}$RB) or "off" (when bound to $_{p}$RB).

7. Raj focused on the differences between HJC-15 hamster glioma cells and U-87 MG human glioma cells.

8. In the hamster glioma cells, Raj found a protein complex that would bind to E2F binding sites. The protein was not identified as any E2F species but rather completely separate proteins referred to Glial E2F1 – associated proteins (GEAPs) having a distinct molecular mass.

9. Although Raj showed that as expected E2F1 would activate E2F-responsive promoters in U-87 MG cell, E2F1 decreased the activity of the promoters in HJC-15.

10. Consequently, Raj provides absolutey no basis for looking at levels of free "E2F."

11. WO 94/18992 to McCormick does not change the situation discussed above.

12. Over time it was proposed that E2F-responsive promoters could be viewed as being in one of three states: (1) fully activated by free E2F, (2) fully repressed by pRB/E2F, or (3) in a basal state (where transcription was driven by non-E2F transcription factors) (See Figure 2). In proliferating cells, pRB would be phosphorylated and E2F-responsive promoters activated/derepressed. In quiescent or resting cells, pRB would be unphosphorylated and E2F-responsive promoters repressed.

13. Multiple groups prior to 1997 showed, in cell culture experiments, that **E2F-responsive promoters** were **more active in proliferating non-transformed cells than quiescent cells** and that mutation of E2F sites typically led to derepression under resting conditions. For example, several groups showed that the promoter for E2F1 itself, which contains 4 E2F sites, was serum-inducible in non-transformed cells such as NIH3T3 cells and REF-52 cells [2-4]. [(2) Johnson, D.G., K. Ohtani, and J.R. Nevins, *Autoregulatory control of E2F1 expression in response to positive and negative regulators of cell cycle progression.* Genes Dev., 1994. **8**: p. 1514-1525; (3) Neuman, E., E.K. Flemington, W.R. Sellers, and W.G. Kaelin, *Transcription of the E2F1 gene is Rendered Cell-Cycle Dependent by E2F DNA-Binding Sites within its Promoter.* Mol. Cell. Biol., 1994. **14**: p. 6607-6615; (4) Hsiao, K.-M., S.L. McMahon, and P. Farnham, *Multiple DNA elements are required for the growth regulation of the mouse E2F1 promoter.* Genes Dev., 1994. **8**: p. 1526-1537.] This was in keeping with the earlier demonstration that E2F1 mRNA accumulates once resting cells are induced to enter S-phase [5, 6] [(5) Kaelin, W.G., W. Krek, W.R. Sellers, J.A. DeCaprio, F. Ajchenbaum, C.S. Fuchs, T. Chittenden, Y. Li, P.J. Farnham, M.A. Blanar, D.M. Livingston, and E.K. Flemington, *Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties.* Cell, 1992. **70**: p. 351-364; (6) Slansky, J.E., Y. Li, W.G. Kaelin, and P.J. Farnham, *A protein synthesis-dependent increase in E2F1 mRNA correlates with growth regulation of the DHFR promoter.* Mol. Cell. Biol., 1993. **13**: p. 1610-1618.] THUS, PRIOR TO OUR WORK, THE EXPECTATION WOULD BE THAT E2F-RESPONSIVE PROMOTERS WOULD BE ACTIVATED IN RAPIDLY PROLIFERATING NORMAL CELLS AS WELL AS IN TUMOR CELLS.

14. The surprising finding we made was that while the E2F1 promoter was very active in tumor cells in vivo, its activity could not be measured in rapidly dividing hepatocytes, which are normal cells. Although the reason for this is still not entirely clear, we did show that absence of promoter activity in normal resting cells relied on E2F-dependent repression and that activity in tumor cells was due, in part, to E2F-dependent activation. Moreover, work done in the mid-late 1990's revealed that E2F1, E2F2, and E2F3, which appear to be the most potent activators in the E2F family, are all encoded by E2F-responsive promoters [2-4, 7, 8] [(2) Johnson, D.G., K. Ohtani, and J.R. Nevins, *Autoregulatory control of E2F1 expression in response to positive and negative regulators of cell cycle progression.* Genes Dev., 1994. **8**: p. 1514-1525; (3) Neuman, E., E.K. Flemington, W.R. Sellers, and W.G. Kaelin, *Transcription of the E2F1 gene is Rendered Cell-Cycle Dependent by E2F DNA-Binding Sites within its Promoter.* Mol. Cell. Biol., 1994. **14**: p. 6607-6615; (4) Hsiao, K.-M., S.L. McMahon, and P. Farnham, *Multiple DNA elements are required for the growth regulation of the mouse E2F1 promoter.* Genes Dev., 1994. **8**: p. 1526-1537; (7) Sears, R., K. Ohtani, and J. Nevins, *Identification of positively and negatively acting elements regulating expression of the E2F2 gene in response to cell growth signals.* Mol Cell Biol, 1997. **17**: p. 5227-5235; (8) Adams, M., R. Sears, F. Nuckolls, G. Leone, and J. Nevins, *Complex transcriptional regulatory mechanisms control expression of the E2F3 locus.* Mol Cell Biol, 2000. **20**: p. 3633-9.].

15. Our unexpected findings appear to be at least partly due to the establishment of a positive feedback loop in tumor cells (with free E2F driving the production of more E2F).

16. Accordingly, we taught there was a selective expression in tumor cells, and that to determine if one would see such an "activated" and thus selective expression, it is important to determine that there is a sufficient amount of E2F present in the cells to result in such a selective

expression. There is simply nothing in these references that in any way suggests or motivates one to look at levels of E2F expression.

17. Our finding has been acknowledged and replicated. See for example Jakubczak, J.L., et al., Cancer Research 63:1490-1499 (2003) wherein they state at page 1490:

> The hypothesis then is that **the increase in free E2F results in an even greater activation** of the E2F-1 promoter in tumor cells with an Rb pathway defect **than in normal** proliferating cells (29). Thus, the promoter for the human E2F-1 gene is an excellent candidate for tumor selective expression of key viral genes controlling viral replication.
>
> The concept of E2F-1 promoter tumor selectively was tested by operably linking the E2F-1 promoter to the *Escherichia* coli β-gal gene in a replication-defective adenovirus. Ad.E2F-βgal (29). Rat livers were efficiently transduced with Ad.E2F-βgal after femoral vein administration, yet no expression of the β-gal gene was detected, as expected. **Surprisingly, the β-gal gene was not expressed in regenerating livers after partial hepatectomy**. In contracts, high levels of β-gal were expressed in rat glioblastoma tumors injected with the Ad.E2F-β-gal adenoviral vector [emphasis added 1490-1491].
>
> **Reference 29, cited here, is Parr, M.J., et al., Nat. Med., 3:1145-1149 (1997), which is the paper that corresponds to the present patent application.**

18. Jakubczak then went on to further confirm our teaching concluding at 1498:

> In conclusion, we have demonstrated that the E2F vector selectively kills a broad range of Rb pathway-defective tumor cells *versus* normal cells. We have shown that the mechanism of this selectively is based on the presence of E2F binding sites within the E2F-1 promoter in the virus and also a disruption of the Rb pathway in the target cell. This characteristic will allow therapeutic broad application of this vector to many cancer types. We also show that the ability of the vectors to replicate is a requirement for full oncolytic activity both *in vitro* and *in vivo*. With systemic delivery, this vector is less toxic than wt and Add*l*1520, additionally indicating its selectivity. Most importantly, we have demonstrated potent antitumor efficacy *in vivo* that is greater than that of Add*l*1520.

19. Accordingly, I respectfully believe that such tumor selectivity, and the importance of looking for E2F expression in malignant cells, is in no way suggested by the prior art references.

20. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

_____

William Kaelin